interactions. The relative rotamer populations of these derivatives were calculated using equations containing terms approximating the dihedral angle, substituent electronegativity, and orientation effects on vicinal coupling constants. As a result of this study, it appears that the dominant factor in this system is the ion–ion or ion–dipole interaction between the carboxylate anion and the imidazolium ion or the polar imidazole ring itself. The coupling constant and chemical shift data have been found to be consistent with a preferred conformation for histidine in basic solution where the carboxyl and imidazole functions are in close proximity. In acidic and isoelectric solutions the ions show equally populated conformations.

Conformation of Cyclic Peptides. VII. Cyclic Hexapeptides Containing the D-Phe-L-Pro Sequence[1]

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Abstract: The cyclic peptides *cyclo*-(L-xxx-D-Phe-L-Pro)$_2$, where xxx is Ala, Orn, or His, were prepared and their conformations were studied by proton magnetic resonance and model building. The effects on the peptide proton resonances of solvent variation, addition of a stable nitroxyl, and, in one case, dilution in chloroform show that the peptide proton of the D-Phe residue is exposed to solvent and that of the L-xxx residue is sequestered, although not completely hidden from small hydrogen bond accepting molecules. Rationalizable differences in peptide solvation by the isomeric butyl alcohols were demonstrated. These observations, together with the observed vicinal coupling constants, indicate no major differences among the backbones of the three peptides. The proposed conformation has the L-xxx residues extended between hairpin turns like those formed by the D-Phe-L-Pro sequence of gramicidin S. The D-Phe-L-Pro peptide bond is trans. χ_1 for both the phenylalanine and histidine side chains is near 180°.

Rational design of peptides with structures suited to particular functions requires predictive power for both backbone and side-chain conformation. To obtain experimental data on which to base predictions about side chains, peptides with well-defined backbones are necessary. Cyclic hexapeptides with C_2 symmetry containing the sequence D-xxx-L-yyy, or its enantiomer, seem likely to possess the desired rigidity.

Empirical estimates of conformational energies indicate that the sequence D-xxx-L-yyy (or its enantiomer) is a particularly favorable one for a sharp reversal of peptide chain direction (a hairpin bend). In making the turn, the first residue can lie in the major region of stability (for a D residue) around $\phi = +100°$, $\psi = -100°$, while the second residue lies in the right-handed helix region of stability (for an L residue) near $\phi = -60°$, $\psi = -60°$.[2-4] Two such turns, connected by two more residues, make up a cyclic peptide that can be expected to have a stable backbone conformation. We have investigated this system, using the sequence D-Phe-L-Pro to form the hairpin bend. The use of proline as the second residue introduces a further backbone constraint, in that ϕ for the proline residue is restricted by the ring structure to a narrow range near −60°. The sequence D-Phe-L-Pro is considered to form hairpin turns in the gramicidin S structure inferred from proton magnetic resonance data.[5,6]

In this work we describe the conformations, obtained by model building in the light of pmr studies, of the cyclic peptides *cyclo*-(L-Ala-D-Phe-L-Pro)$_2$ (**1**), *cyclo*-(L-His-D-Phe-L-Pro)$_2$ (**2**), and *cyclo*-(L-Orn-D-Phe-L-Pro)$_2$ (**3**). A study of the analog, *cyclo*-(Gly-D-Phe-L-Pro)$_2$, has been completed by Blout, Deber, and Pease.[7]

The high solubility of **1** in organic solvents permitted an inquiry into its self-association in chloroform and an examination of the solvation of its peptide protons by alcohols of varying steric requirements, in addition to studies using the more usual solvents.

Experimental Section

Proton magnetic resonance spectra were obtained using the 250-MHz spectrometer of the NMR Facility for Biomedical Research at Carnegie-Mellon University.[1] Resonances were assigned to amino acid residues in the usual manner, using spin decoupling in frequency sweep operation.

Materials. Except for hexafluoro-2-propanol-d_2, solvents for the pmr work were commercial products used without further purification. The dimethyl-d_6 sulfoxide was the nominal 100.0% material of Diaprep Inc. Hexafluoro-2-propanol-d_2 was prepared by high-pressure deuteration of hexafluoroacetone over Adams

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(7) E. R. Blout, C. M. Deber, and L. G. Pease, personal communication.

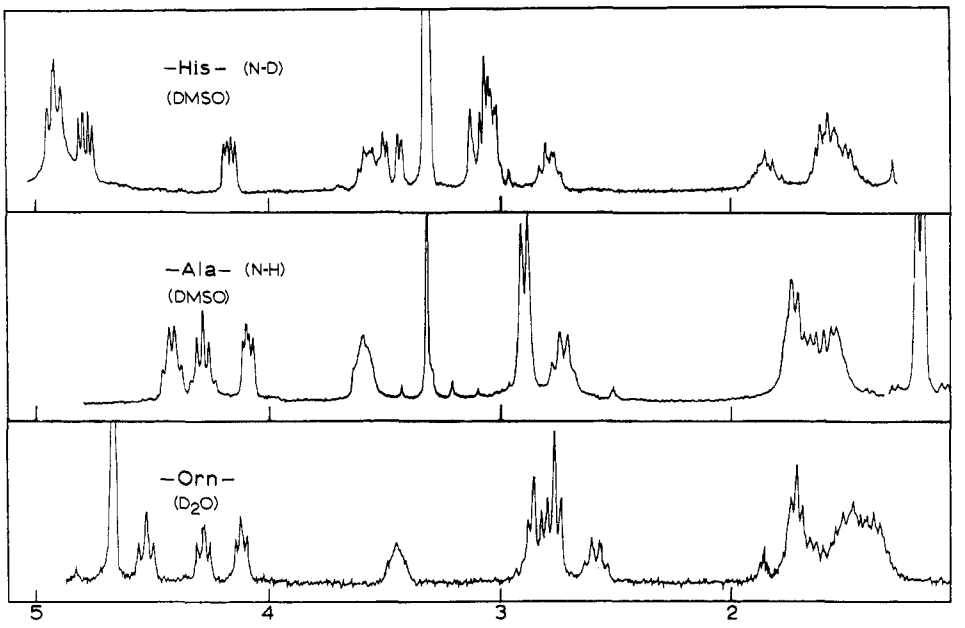

Figure 1. Upper field regions of the 250-MHz proton magnetic resonance spectra of the peptides *cyclo*-(L-xxx-D-Phe-L-Pro)$_2$. Reference is tetramethylsilane for the dimethyl-d_6 sulfoxide (nominal 100%) solutions, and capillary hexamethyldisiloxane for the aqueous solution. Peptide concentrations are about 50 mg/ml.

catalyst.[8] We thank Mr. D. R. Pilipauskas for the preparation. 3-Oxyl-2,2,4,4-tetramethyloxazolidine was prepared as described earlier.[9]

The linear precursors of the cyclic peptides were prepared by stepwise synthesis of tripeptide fragments that were coupled to give linear hexapeptides. The intermediates were not thoroughly purified, but thin-layer chromatography and proton magnetic resonance analyses were used to ensure that each intermediate had the required composition before the next step was undertaken. The cyclization steps, purifications, and characterizations of the final products are described below.

cyclo-(L-Orn-D-Phe-L-Pro)$_2$ (3). Crude H-(N$^\delta$-Boc-L-Orn-D-Phe-L-Pro)$_2$-NHNH$_2$ (3 g, *ca.* 3.2 mmol) in 10 ml of purified[10] dimethylformamide (DMF) was cooled to −40° and stirred during the addition of, first, 8.55 ml of freshly prepared and standardized 1.85 *N* hydrogen chloride in tetrahydrofuran and then 0.51 ml (3.8 mmol) of isoamyl nitrite. After 30 min at −40°, when a spot test for residual hydrazide (5% ferric chloride plus 5% potassium ferricyanide) was negative, 350 ml of chilled purified DMF containing 2.64 ml of triethylamine (19 mmol) was added. The mixture was stored and stirred at −10° for 2 days.

The solvent was removed under vacuum and the residue was triturated with water and, upon solidification, was washed thoroughly with water. After drying under vacuum, the mixture of products was dissolved in about 30 ml of anhydrous trifluoroacetic acid and stored 1 hr at room temperature to remove the N$^\delta$-butoxycarbonyl groups. The trifluoroacetic acid was evaporated under reduced pressure and the residue was triturated with several changes of ether.

The now unblocked cyclic peptide mixture was chromatographed on Sephadex G-15 in 2% aqueous acetic acid. Apart from the major component, a higher molecular weight fraction was also obtained. The major component, which had the proper elution volume for hexapeptide, was converted to the free base by passage in 20% water in methanol through a bed of AG-1 X4 (Bio-Rad) strong base anion exchange resin in the hydroxide form. It was then passed in ethanol through a charcoal bed and lyophilized from water to give 0.84 g (41%) of a white, chromatographically homogeneous (thin-layer chromatography, two solvent systems) product. The higher molecular weight fraction was similarly converted to the free base. It was water insoluble and amounted to 0.17 g.

For characterization the crystalline hydrochloride of the cyclic hexapeptide was prepared by treatment with hydrogen chloride in ether.

Anal. Calcd for $C_{38}H_{52}N_8O_6 \cdot 2HCl \cdot 3H_2O$: C, 54.09; H, 7.17; N, 13.28; Cl, 8.40. Found: C, 53.84, 54.26; H, 6.81, 6.87; N, 13.31, 13.90; Cl, 8.77.

The proton magnetic resonance spectrum of the cyclic hexapeptide (Figure 1), upon integration, indicated the proper ratios of the various kinds of protons and possessed no extraneous resonances other than those of water protons.

A sample of the peptide was converted to the bis(N$^\delta$-2,4-dinitrophenyl) derivative by treatment with 2,4-dinitrofluorobenzene in aqueous bicarbonate. The chromatographically homogeneous product was hydrolyzed, and the hydrolysate was demonstrated, by comparison with an authentic sample on thin-layer chromatography, to contain N$^\delta$-dinitrophenylornithine as the only dinitrophenyl derivative present.

cyclo-(L-Ala-D-Phe-L-Pro)$_2$ (1). Crude H-(L-Ala-D-Phe-L-Pro)$_2$-NHNH$_2$ (4 g, *ca.* 3.2 mmol) was cyclized by the procedure described above for the preparation of 3. The 1.37 g of water-insoluble solid obtained after removal of cyclization solvent and trituration with water was dissolved in methanol containing 25% water and passed through a bed of Baker M-614 mixed bed (H^+/OH^- form) ion exchange resin. The material passing through the resin was chromatographed on Sephadex LH-20 in ethanol containing 20% water. A chromatographically homogeneous (thin-layer chromatography in several solvent systems), ether-soluble, ninhydrin negative fraction, 0.22 g, was obtained. In addition, 0.6 g of ether-insoluble material of higher molecular weight was also isolated. Both of these substances had pmr spectra characteristic of the composition (Ala, Phe, Pro), but the ether-insoluble material had the broad lines characteristic of higher molecular weight material. The spectrum of the ether-soluble material showed only the lines of Phe, Ala, and Pro residues, in the proper ratios (Figure 1).

The ether-soluble product was characterized as the cyclic hexapeptide by its mass spectrum. This spectrum showed the parent ion at *m/e* 630; no other peaks above *m/e* 120 were so intense. A peak at *m/e* 447, one-fifth the parent intensity, corresponding to peptide less Pro, Ala, and NH fragments, was the next most intense above *m/e* 400, although small amounts (<0.1 parent intensity) of other ions corresponding to recognized cyclic peptide fragmentations[11] were apparent. Significant amounts (>0.3 parent) of ions at *m/e* 371 (parent less Phe, Pro, and NH), 315 (one-half of the peptide), and 300 (one-half the peptide, less NH) were observed.

cyclo-(L-His-D-Phe-L-Pro)$_2$ (2). Crude N$^\alpha$,N^{im}-Z$_2$-L-His-D-Phe-L-

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Table I. Proton Chemical Shifts and Coupling Constants of *cyclo*-(L-XXX-D-Phe-L-Pro)$_2$[a]

XXX	Solvent	D-Phe δ_{NH}	D-Phe $J_{\alpha NH}$	D-Phe δ_α	D-Phe $J_{\alpha\beta}$	D-Phe δ_β	L-Pro δ_α	L-Pro $J_{\alpha\beta}$	L-Pro δ_δ	L-Pro δ_δ	L-XXX δ_{NH}	L-XXX $J_{\alpha NH}$	L-XXX δ_α	L-XXX $J_{\alpha\beta}$	L-XXX δ_β
His	DMSO	7.72	6.3	4.58	6.3	2.86	4.10	3.8	2.75	3.4[b]	7.2[b]		4.46	9.3	2.84
					9.3	2.90		8.2						4.6	3.02
His	MeOH	8.05	6.5	4.79		3.02	4.17	4	2.70	3.53	7.70	8.3	4.65	9.9	3.06
						3.02		8.5						5.4	3.23
His (Trifluoroacetate)	MeOH	8.16	7.3	4.92	9.3	3.02	4.17	4	2.79	3.47	8.32	8.7	4.79	10.5	3.06
					5.9	3.06		8.5						4.5	3.45
Orn	DMSO	8.27	⩾4	4.53	6	2.88	4.13	Σ = 12	2.84	3.60	7.45	7	4.22		
					9	2.94									
Orn (Acetate)	H_2O	8.77	5	5.01	9.8	3.30	4.60	4.5	3.05	3.92	7.90	8.5	4.77		
					6.2	3.36		8							
Ala	DMSO	8.48	5.3	4.41		2.90	4.08	4	2.73	3.68	6.96	6.3	4.28	6.4	1.17
						2.90		7							
Ala	MeOH	8.29	6.3	4.75	6.1	3.02	4.20	Σ = 11	2.80	3.66	7.66	7.0	4.34	7	1.35
					9.9	3.06									
Ala	$CDCl_3$	7.34[c]	6	4.51[b]	5.6	2.99	4.51[b]		2.60	3.72	6.96[c]	8.3	4.58	6.6	1.35
					10.5	3.16									

[a] Coupling constants are in hertz, chemical shifts are ppm below internal tetramethylsilane, except that the reference is capillary hexamethyldisiloxane for the aqueous solution. Peptide concentration is about 50 mg/ml, temperature 30°. [b] Resonance obscured by another. [c] Concentration dependent; values are given for 7 mg/ml.

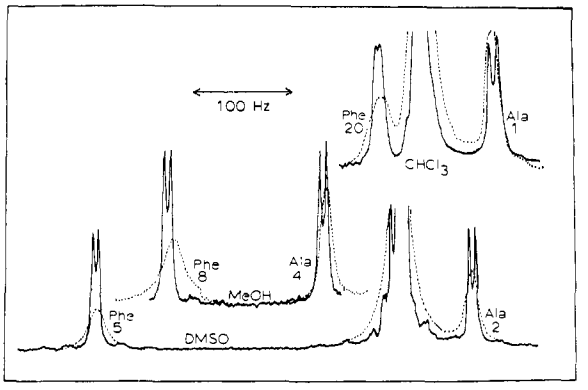

Figure 2. Effect of added nitroxyl on peptide proton line widths of *cyclo*-(L-Ala-D-Phe-L-Pro)$_2$. Spectra are given for solutions without added radical (—) and with 2% radical in the dimethyl sulfoxide and methanol solutions and 1% in the chloroform solution (- - - -). The numerals are the approximate line-width increments produced by the radical, hertz at half-height per per cent radical. Peptide concentration is about 50 mg/ml. Superimposed spectra were recorded with the same operating parameters and are aligned with respect to the reference, but the positions of pairs relative to each other are only approximately correct.

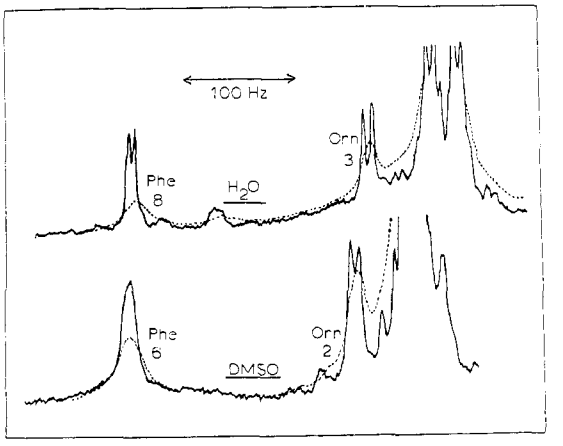

Figure 3. Effect of added nitroxyl on peptide proton line widths of *cyclo*-(L-Orn-D-Phe-L-Pro)$_2$. Spectra are given for solutions without added radical (—) and with 2% radical (- - - - -). Peptide concentration is about 50 mg/ml. The numerals are the approximate line-width increments produced by the radical, hertz at half-height per per cent radical. The spectra are aligned with respect to the reference (capillary hexamethyldisiloxane for the aqueous solution). The aqueous solution contains 5% acetic acid-d_3.

Pro-L-His-D-Phe-L-Pro-*O*-*t*-Bu (9.5 g, *ca.* 8.6 mmol) was treated for 1 hr with 12 g (5 equiv) of 30% hydrogen bromide in acetic acid, and the resulting hydrobromide was precipitated by addition of a large volume of anhydrous ether. The precipitate was washed thoroughly with ether and stored under vacuum over phosphorus pentoxide and potassium hydroxide.

The unblocked product, 7.7 g (7.4 mmol), was dissolved in 150 ml of purified DMF. Triethylamine (3.1 ml, 22 mmol), followed by 6 g (31 mmol) of *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride, was added. The reaction mixture was stirred at room temperature overnight.

The cyclization solvent was removed under vacuum. The residue was subjected to countercurrent distribution (50 transfers, 100 ml per phase) between ethyl acetate and water to remove the bulk of the urea and unreacted carbodiimide. The contents of the tubes containing the cyclic hexapeptide (identified by R_f value and by negative ninhydrin and positive Pauly tests on thin-layer chromatography) were chromatographed (2% aqueous acetic acid) on Sephadex G-15. The cyclic hexapeptide fraction was converted to the free base by passage, in 50% aqueous methanol, through Bio-Rad AG-3 X4 weak base resin (OH^- form) and then through a charcoal bed. It was crystallized from methanol–water and dried at 100° under 0.05 mm pressure for 2 days; 290 mg was obtained, 4.3% overall. This product was homogeneous on thin-layer chromatography in several solvent systems.

The proton magentic resonance spectrum of this product was consistent with the composition (His, Phe, Pro), with no extraneous resonances other than readily exchangeable water (Figure 1).

Anal. Calcd for $C_{40}H_{46}N_{10}O_6 \cdot 2.5H_2O$: C, 59.47; H, 6.36; N, 17.34. Found: C, 59.29; H, 6.05; N, 17.28.

Results

Tables I–IV and Figures 1–3 present the pmr data collected in this study. They may be summarized as follows.

1. The peptides have C_2 symmetry in the nmr time average, as indicated by the representative spectra shown in Figure 1, which show that only one α-proton resonance appears for each kind of amino acid residue. All resonances are accounted for by a single component. There is no evidence for slow exchange between two forms differing in cis–trans isomerism about the Phe-Pro bond.[12–14]

(12) C. M. Deber, F. A. Bovey, J. P. Carver, and E. R. Blout, *J. Amer. Chem. Soc.*, **92**, 6191 (1970).
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(14) D. A. Torchia, S. C. K. Wong, C. M. Deber, and E. R. Blout, *J. Amer. Chem. Soc.*, **94**, 616 (1972).

Table II. Proton Chemical Shifts of *cyclo*-(L-xxx-D-Phe-L-Pro)$_2$ in Dimethyl Sulfoxide and Hexafluoro-2-propanol[a]

xxx	Solvent	Phe NH	Phe α	Phe β[b]	Pro α	Pro δ	Pro δ	X NH	X α	X β[b]
Ala	DMSO-d_6	8.48	4.41	2.90	4.08	2.73	3.68	6.96	4.28	1.17
	HFP-d_2	7.10	4.63	3.07	4.34	2.50	3.67	7.13	4.50	1.39
His	DMSO-d_6	7.72	4.58	2.88	4.40	2.75	3.4	7.2[d]	4.46	2.93
	HFP	7.06	4.7[c]	2.93	4.44	2.54	3.53	7.3[d]	4.7[c]	3.00
Orn	DMSO-d_6	8.27	4.53	2.91	4.13	2.84	3.60	7.45	4.13	
	HFP	7.03	4.55[c]	3.07	4.35	2.61	3.65	7.3[d]	4.55[c]	

[a] Chemical shifts are given in ppm below internal tetramethylsilane, at 30°, peptide concentration about 50 mg/ml. [b] Mean position of β protons is given. [c] α protons overlap. [d] Under aromatic proton absorption.

Table III. Peptide Proton Resonances of *cyclo*-(L-Ala-D-Phe-L-Pro) in Chloroform as a Function of Concentration, 21°

Concn, mg/ml	Chemical shift, ppm, TMS reference: Phe NH	Ala NH	$CHCl_3$	ArH of Phe
240	7.73	7.05	7.35	7.24
140	7.57	7.01	7.31	7.25
70	7.48	6.99	7.29	7.25
30	7.42	6.98	7.26	7.26
20	7.37	6.97	7.26	7.26
7	7.34	6.96	7.26	7.26

Table IV. Peptide Proton Resonances of *cyclo*-(L-Ala-D-Phe-L-Pro)$_2$ in Alcohols

Solvent	Bp, °C	Ar-H[a]	Phe[a]	Ala[a]	AcNHMe[b]
n-BuOH	118	7.25	8.17	7.90	7.97
i-BuOH	108	7.23	8.26	7.73	7.97
s-BuOH	100	7.27	8.37	7.65	7.97
t-BuOH	82	7.24	8.39	7.54	7.90
EtOH		7.26	8.32	7.81	

[a] Chemical shifts in ppm below tetramethylsilane; peptide concentration about 80 mg/ml, temperature 21°. For the aromatic protons the chemical shift of the maximum of the envelope is given. [b] *N*-Methylacetamide concentration 10 mg/ml, temperature 35°.

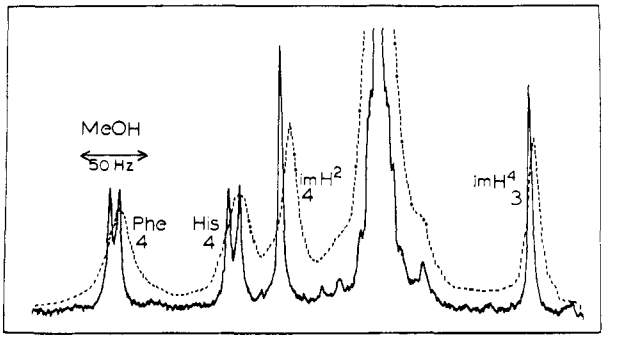

Figure 4. Effect of added nitroxyl on peptide proton line widths of *cyclo*-(L-His-D-Phe-L-Pro)$_2$. The broken line is the spectrum of a solution containing 3% radical and is aligned with the solid line spectrum of the radical free solution, but was recorded at twice the amplification. The numerals are the approximate line-width increments produced by the radical, hertz at half-height per per cent radical.

2. In proton-accepting solvents the peptide proton resonance of the variable residue is at higher field than the phenylalanine peptide proton, usually near the aromatic proton resonances.

3. According to a variety of criteria, the peptide proton of the variable residue is less exposed to solvent than is the peptide proton of the phenylalanine residue.

4. The H_α–H_N vicinal coupling constants suggest dihedral angles of 150 ± 10° for the variable residue and 130 ± 10° for the phenylalanine.

5. The Phe-Pro peptide bond is trans (ω = 180°) according to a correlation suggested by Patel:[15] the H_α–H_β vicinal couplings of the proline residue are 4 and 8 Hz, rather than *ca.* 0 and 8 Hz.

The above observations can be accommodated in a single model for all three peptides. Details of the observations and the model are discussed below.

Solvent Exposure of Peptide Protons. Peptide proton solvent exposure was determined without the aid of temperature variation studies. The principal probes employed were the effects on chemical shift of changing from good proton accepting solvents (dimethyl sulfoxide, methanol) to a poor proton accepting solvent (hexafluoro-2-propanol),[16,17] and the differential effect on line width of a hydrogen bonding free radical additive.[9,17]

In Table II the proton chemical shift values for cyclic peptides **1**, **2**, and **3** in dimethyl sulfoxide (DMSO) are compared with the values for hexafluoro-2-propanol (HFP) solutions. The peptide protons of *cyclo*-(L-Ala-D-Phe-L-Pro)$_2$ do not undergo exchange with HFP-d_2 over many hours, an indication of its low basicity. The peptide protons of **2** and **3**, which have basic side chains, do exchange rapidly in this solvent, however. On going from DMSO to HFP the chemical shifts of carbon bound protons change ±0.2 ppm; this is also about the change observed for the peptide protons of the alanine, histidine, or ornithine residues. The principal effect of the solvent change is that the phenylalanine peptide proton resonance moves upfield 1.2–1.4 ppm in **1** and **3**, and upfield 0.7 ppm in the histidine peptide, **2**. This large shift brings the phenylalanine peptide proton resonance (in HFP) to about 7.1 ppm; in chloroform, another nonbasic solvent, the corresponding chemical shift is about 7.3 ppm, measured for the chloroform-soluble alanine peptide, **1** (Table III). From these data, it can be concluded that the peptide proton of the variable residue is shielded from dimethyl sulfoxide, and the phenylalanine peptide proton is exposed, although to a reduced extent in the histidine case, **2**.

Single-solvent data consistent with the same conclusion are the effects of dilution in chloroform on the spectrum of peptide **1** (Table III). As the solution

(15) D. J. Patel, *Biochemistry*, 12, 667 (1973).
(16) T. P. Pitner and D. W. Urry, *J. Amer. Chem. Soc.*, 94, 1399 (1972).
(17) K. D. Kopple and T. J. Schamper in "Chemistry and Biology of Peptides," Proceedings of the Third American Peptide Symposium, Boston, Mass., June, 1972, J. Meienhofer, Ed., Ann Arbor Science Publishers, Ann Arbor, Mich., 1972, p 75.

becomes more concentrated, the phenylalanine proton resonance moves downfield, suggesting hydrogen bonding by self-association. Although the alanine peptide proton also moves downfield, it does so much less rapidly, with about the dependence of the weak proton donor, chloroform. A completely nonbonding reference, the aromatic protons of the phenylalanine, is almost independent of concentration.

Figures 2–4 give another indication of the differing solvent exposure of the two kinds of peptide proton. They illustrate effects on spectra of the cyclic peptides of 3-oxyl-2,2,4,4-tetramethyloxazolidine, a stable free radical that acts as a hydrogen bond proton acceptor.[9,17] Spectra in the peptide proton region were examined upon addition of 1, 2, and 3% (by volume) radicals, and estimates of the incremental increase in width at half maximum height of the N–H doublets were made. Such quantitative estimates are subject to large errors because of overlaps, but the differential effects themselves are visible in the superimposed spectra of the figures. In the alanine (**1**) and ornithine (**3**) peptides the phenylalanine peptide proton line is visibly more broadened by the addend than is the second peptide proton. There is no apparent difference in the histidine (**2**) case, in agreement with the smaller solvent shift mentioned previously. The increasing incremental broadening of the phenylalanine proton line of **1** in the order DMSO, methanol, chloroform (Figure 2) may be an indication of decreased competition by the solvent for hydrogen bonding to the exposed peptide proton.

In considering the effects, through hydrogen bonding, of additives or solvent changes on peptide protons resonances, it should be remembered that the basic center of the additive or solvent is variously hindered by the groups to which it is attached. In the absence of conformation changes, a peptide proton that is exposed to one solvent may appear shielded to another. Access to oxygen is increasingly hindered in the sequence methanol, dimethyl sulfoxide, di-*tert*-alkyl nitroxyl. Thus the phenylalanine peptide proton of **2**, which is no more exposed than the histidine proton according to the nitroxyl experiment, is somewhat exposed by the DMSO–HFP shift criterion. A related observation is that the resonances of the supposedly sequestered protons of the alanine and *uncharged* histidine residues in **1** and **2** move downfield on changing from DMSO to methanol (Table I). This suggests that they become somewhat associated with solvent in the smaller solvent. Similar downfield shifts of sequestered protons on changing from DMSO to methanol have been observed before.[18]

At constant conformation, the shift in a peptide proton resonance with solvent change ought to depend on both solvent shape and solvent basicity. To give an indication of the effect of solvent bulk, we examined the shifts of the peptide protons of **1** in a series of solvents of the same basicity and similar molar volume, the butyl alcohols. Table IV indicates the result. While the resonance position of the amide proton *N*-methylacetamide is almost unaffected, the alanine peptide proton moves upfield 0.36 ppm on going from normal to *tert*-butyl alcohol and, surprisingly, the phenylalanine proton moves downfield 0.22 ppm. The resonances of the aromatic protons of the phenylalanine side chain are virtually unaffected by the solvent variations, indicating the absence of nonspecific solvent effects unrelated to hydrogen bonding. Assuming a single conformation in all the alcohols (consistent with observed almost invariant H_α–H_N coupling constants), these observations indicate that the alanine proton is more strongly bonded to solvent in the less hindered alcohols, as expected, while the phenylalanine peptide proton may be specifically solvated by the secondary and tertiary alcohols. The latter interpretation fits well with the conformation we suggest below. If a change in solvation of the alanine proton is the source of its change in chemical shift, as we believe, it is unlikely that this proton is involved in a strong intramolecular hydrogen bond.

Peptide proton resonance temperature coefficients were determined for the ornithine peptide, **3**, in aqueous solution. The phenylalanine proton shifts upfield 0.007 ppm/degree, and the ornithine proton 0.004 ppm/degree. The indication here is that the ornithine peptide proton is not completely sequestered from water. However, Blout, Deber, and Pease[7] measured the temperature coefficients for **1** in dimethyl sulfoxide and obtained 0.0068 and 0.0017 ppm/degree for the phenylalanine and alanine protons, respectively. These results can be taken to indicate that the alanine peptide proton is largely shielded from dimethyl sulfoxide.

Backbone Dihedral Angles. The observed H_α–H_N vicinal coupling constants of phenylalanine in the three peptides range from over 4 to about 6.5 Hz (omitting the cationic form of **2** in methanol). The corresponding values for the variable residue are 6.5–8.5 Hz. These correspond to dihedral angle ranges of 130 ± 10 and 150 ± 10°, respectively.[19] The range 20 ± 20° for the phenylalanine, although consistent with the coupling constants, is ruled out by other constraints introduced in the model building. For a model representative of the three peptides, the phenylalanine dihedral angle is taken as 130° and the variable residue dihedral angle as 150°.

There are always doubts about the use of nmr derived dihedral angles in model building. First, there is the fact that the coupling constants are time-average values, and a conformation based on them is likely only if the peptide backbone occupies but one region of conformation space. The constraints introduced by the D-Phe-L-Pro combination make this likely in the present cases. Beyond the averaging problem, however, there are questions about the planarity of the peptide bond. Although planarity is regularly assumed in model construction, peptide bonds need not necessarily be exactly cis or trans. The rms deviation from 180° in the reported ω angles for tosyl-α-chymotrypsin[20] is 6°. Further, the trigonal nature of the peptide nitrogen has been called into question,[21,22] which not only increases doubts about the relationship between the N–H and C–O vectors, but should

(18) K. D. Kopple, A. Go, R. H. Logan, Jr., and J. Savrda, *J. Amer. Chem. Soc.*, **94**, 973 (1972).

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(22) F. K. Winkler and J. D. Dunitz, *J. Mol. Biol.*, **59**, 1971 (1969).

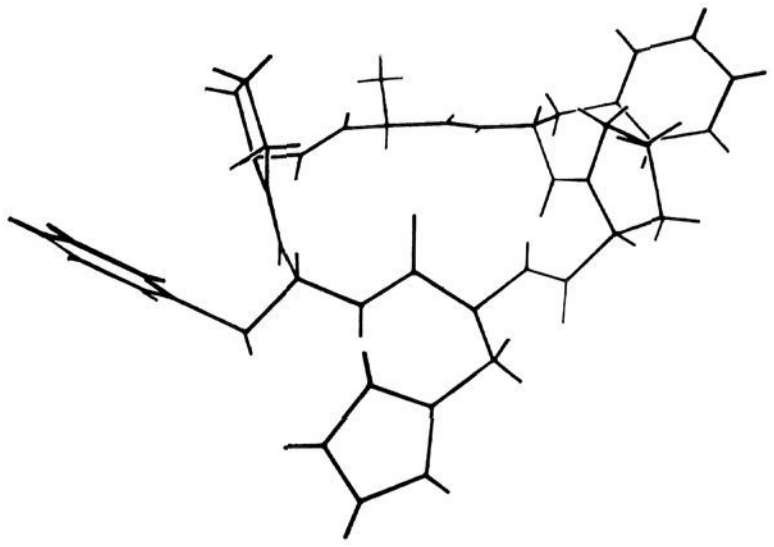

Figure 5. Model proposed for *cyclo*-(L-XXX-D-Phe-L-Pro)$_2$. The xxx residue is shown as histidine on the near side and alanine on the far side. The dihedral angles are: L-xxx, $\phi = -150°$, $\psi = 170°$; D-Phe, $\phi = 70°$, $\psi = -120°$, χ_1, *ca.* 180°, χ_2, *ca.* 90°, L-Pro, $\phi = -60°$, $\psi = -60°$. χ_1 for L-His is *ca.* 180°. This is a photograph of a Kendrew model, retouched to eliminate the barrel connectors.

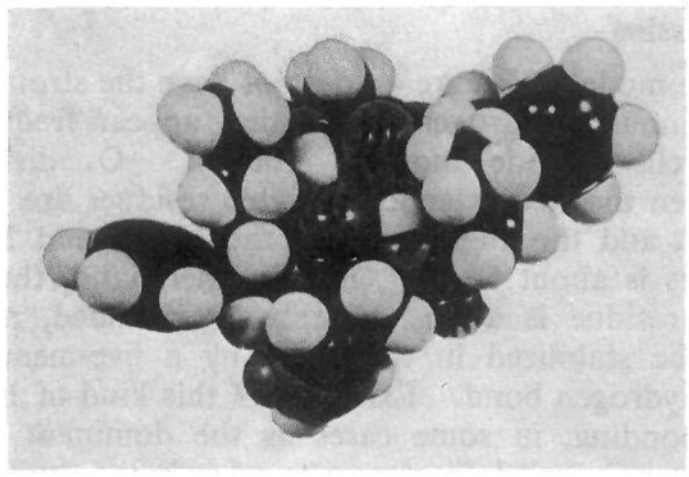

Figure 6. CPK model of the conformation shown in Figure 5.

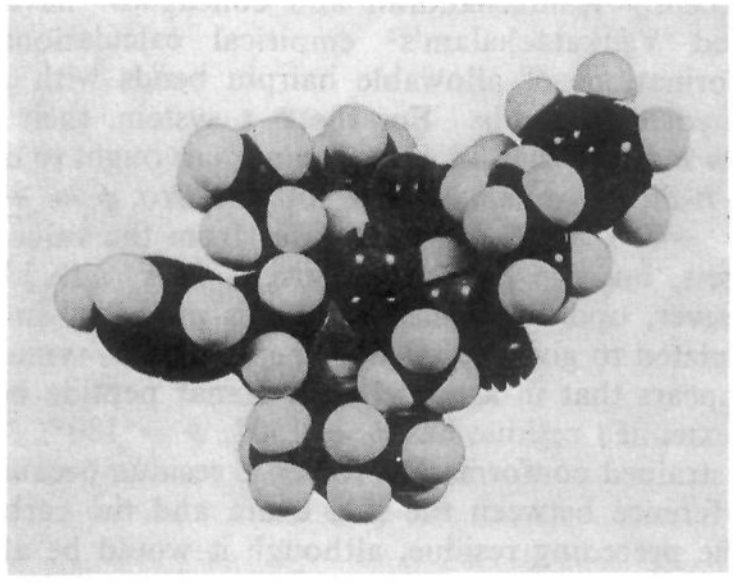

Figure 7. Model of *cyclo*-(L-Ala-D-Phe-L-Pro)$_2$ specifically associated with 2-butanol as suggested in the text.

also reduce faith in the validity of any coupling constant–dihedral angle correlation. These problems cannot be resolved with available data, but they are again raised here as a caveat.

Suggested Backbone Conformation. All of the experimental constraints, the estimated dihedral angles, the cyclic nature of the peptides, the apparent C_2 symmetry, the probable trans configuration of the Phe-Pro peptide bond, the restriction of ϕ for L-proline to values near $-60°$, the solvent shielding of the peptide proton of the variable residue, and the solvent exposure of the phenylalanine proton in two of the three cases (the exception will be subsequently explained) can be combined with standard bond angles and distances (planar amide groups) to obtain a most likely backbone conformation. This has the conventional[23] angles: D-Phe, $\phi = 70°$, $\psi = -120°$; L-Pro, $\phi = -60°$, $\psi = -60°$; L-xxx, $\phi = -150°$, $\psi = 170°$. This conformation is illustrated in Figures 5 and 6.

Side-Chain Rotations. The H_α–H_β vicinal coupling constants of the phenylalanine are uniformly near 6 and 10 Hz, and the chemical shift difference between the two β protons is small, generally less than 0.1 ppm (Table I). The coupling constants could correspond[24] to something near an eclipsed conformation, $\chi_1 = 0$ or $-120°$, but this is improbable. More likely, they indicate either a distribution among torsional minima with the predominant rotamer near $\chi_1 = -180$ or 60°, or else a single rotamer, either $\chi_1 = -150°$ or $\chi_1 = 30°$. (Note that the phenylalanine is of the D series.)

A choice of the range of χ_1 is readily made. Table I indicates that one of the δ protons of the proline residue is shifted 0.8 ppm upfield from the usual proline δ resonance near 3.5 ppm. This is a likely effect of the magnetic anisotropy of the aromatic ring, and fits well, given the backbone already indicated, with χ_1 in the 180° region, while excluding the alternative.

The small chemical shift difference between the two β protons of the phenylalanine may be quite tentatively interpreted as indicating that they are similarly affected by the aromatic ring current. For this χ_2 should be close to 90°, and this is consistent also, if χ_1 is near 180°, with the effect of the aromatic ring on the proline δ proton.

Given the position of the D-phenylalanine side chain just suggested, models offer an explanation for possible specific solvation of the phenylalanine peptide proton of **1** by *sec*- and *tert*-butyl alcohols. Such solvation occurs with a very good fit, maximizing van der Waals interactions, of the flanking methylenes or methyls of the carbinol with the α and β groups of the alanine on one side and with the β methylene of the phenylalanine on the other, as illustrated in Figure 7. Only half this interaction is likely with butyl alcohol or isobutyl alcohol.

The H_α–H_β couplings of the histidine residue in **2** suggest that its side chain has a favorable position near either $\chi_1 = -60°$ or $\chi_1 = 180°$. As with the phenylalanine, the choice is in favor of the region near 180°. Space filling models indicate that at $\chi_1 = -60°$ the imidazole ring is crowded by the proline carbonyl and β methylene. In addition, with $\chi_1 = 180°$ there is proximity of the imidazole to the phenylalanine peptide proton, and thus reason for the absence of association between that proton and the stable free radical (Figure 3). The relatively upfield position of the phenylalanine proton of **2** in DMSO solution, in comparison with peptides **1** and **3**, also suggests that its solvation is somewhat inhibited.

Our data do not suggest to us conformation for the ornithine side chain of **3**.

(23) IUPAC–IUB Commission on Biochemical Nomenclature, *Biochemistry*, **9**, 3471 (1970).

(24) We use $J(\text{Hz}) = 11.0\cos^2\theta - 1.4\cos\theta + 1.5\sin^2\theta$ for estimating dihedral angles from coupling constants in the H_α–H_β system: K. D. Kopple, G. R. Wiley, and R. Tauke, *Biopolymers*, **12**, 627 (1973).

Discussion

The model of Figure 4 does not have the strong 1,4-transannular hydrogen bonds that appear frequently in cyclic peptide models. The $N_1 \cdots O_4$ distances between the two extended variable residues are about 3.8 Å, and the angle between the N_1–H_1 and N_1–O_3 vectors is about 50.°[25] On the other hand, the variable residue is almost completely extended, and it may be stabilized in this form by a five-membered ring hydrogen bond. Existence of this kind of hydrogen bonding, in some cases as the dominant form, has been inferred from results of infrared studies of *N*-acyl amino acid amides in dilute carbon tetrachloride solution.[26-28]

Recently Ramachandran and colleagues[4] have extended Venkatachalam's[2] empirical calculations of conformations of allowable hairpin bends with good 1,4 hydrogen bonds. For the D–L system, their estimates indicate that an energy minimum ought to occur near D-Phe, $\phi = 60°$, $\psi = -100°$; L-Pro, $\phi = -60°$, $\psi = -40°$. This is not very far from the values we suggest, but the N_1–O_4 distance is 3 Å, not 3.8 Å. However, upon examination of this model when it is completed to give a cyclic hexapeptide of C_2 symmetry it appears that in a model with planar peptide bonds the extended residue has $\phi = 150°$, $\psi = 180°$. This is a strained conformation for an L residue because of interference between the side chain and the carbonyl of the preceding residue, although it would be allowable for glycine.

Model building from nmr data does not take into account the small distortions of bond angles and departures from planarity which must occur to minimize the total energy of the actual peptide. The sum of these minor adjustments could result in formation of 1,4 hydrogen bonds in the real peptide. However, experimental demonstration (data of Table IV) of partial exposure of the peptide protons of the variable residue argues against their involvement in strong intramolecular interactions. In our model, hindrance to solvation of the protons of the variable residue arises from the proline ring and from the side chain of the variable residue itself.

The preferred side-chain rotation of the phenylalanine residue in the present set of peptides, χ_1 near 180°, is the same as that suggested earlier for the tyrosine residues in *cyclo*-(Gly-L-Tyr-Gly)$_2$, *cyclo*-(Gly$_5$-L-Tyr), and related peptides in which the aromatic residue has $\phi = -80°$, $\psi = 120°$, *i.e.*, the same backbone conformation.[18,29] In that the aromatic ring of the side chain achieves proximity to the π system of a peptide carbonyl, this is analogous to the folded conformations found for aromatic side chains in cyclic dipeptides.[30,31]

We do not detect much in the way of clear systematic effects of side-chain variation (in the variable residue) on the peptide backbones of **1**, **2**, and **3**. Minor changes are reflected in the H_α–H_β coupling constants. Major changes would probably also affect solvent exposure and chemical shift of peptide protons. The only obvious effect of the latter type, reduced solvation of the phenylalanine proton in **2**, is explained by the bulk and position of the histidine side chain. Although there is coupling constant variability, the effects produced by solvent change are as large as those produced by side-chain variation. H_α–H_β coupling constants are very sensitive to dihedral angle in the range 110–160°, so that the observed changes correspond to small changes in ϕ. For most of these we cannot see obvious reasons. There does seem to be a 15–20° increase in the phenylalanine H–N–C_α–H angles of **2** relative to the ornithine peptide **3**. This might occur to relieve crowding of the phenylalanine N–H with the histidine side chain in its preferred 180° conformation. Beyond this tentative suggestion, we conclude that the backbone is chiefly determined by the D-Phe-L-Pro sequence and the fact that the third residue has a β carbon and is of the L series.

The hairpin bend described for the present set of peptides has the proline residue at its C side, position 3 in Venkatachalam's[2] numbering. This is also the case for the D-Phe-L-Pro sequences in gramicidin S,[5,6] but it is not so for the peptides *cyclo*-(Gly-L-Pro-L-Ser)$_2$ and *cyclo*-(L-Ser-L-Pro-Gly)$_2$. In those conformations of these last two peptides that have trans XXX-L-Pro bonds, the proline is at position 2, the N side of the bend.[13,14] The corresponding conformation for *cyclo*-(L-XXX-D-Phe-L-Pro)$_2$ would have to have a L–L hairpin turn analogous to that proposed for *cyclo*-(Gly-L-Pro-L-Ser)$_2$. It is conceivable that in *cyclo*-(Gly-L-Pro-L-Ser)$_2$, the L–L turn is stabilized by hydrogen bonding involving the serine hydroxyl; in the present cases such stabilization would be absent.

(25) A referee points out that the peptide molecule is not static but is moving about in a region of low conformational energy, and that conformations with shorter and more linear hydrogen bonds may contribute to the stabilization of that region. We suggest that if such contributions are important, the nmr averages should lead to models with shorter N–H $\cdots$ O distances and smaller deviations from linearity. Anticipating a report to be made later, we report that the retro isomer of **1** has one conformation for which the model constructed from the nmr data contains a good (short, more linear) 1,4 hydrogen bond. In this case the proton involved comes into resonance at 8.2 ppm in DMSO, 8.3 ppm in HFP, a hydrogen bond induced shift downfield 1.2 ppm from the internal proton of **1**.

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